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FORM PTO-1390 (Modified) (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER: 38331-0002

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5): 09/856640

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/06528	22 September 2000 (22.09.00)	30 September 1999 (30.09.99)

TITLE OF INVENTION

A PIGMENT-CONTAINING SUBSTANCE FOR FEED ADDITIVES

APPLICANT(S) FOR DO/EO/US

Akira TSUBOKURA, Hisashi YONEDA and Haruyoshi MIZUTA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. X This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.

2. __ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

3. ___ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ___ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. X A copy of the International Application as filed (35 U.S.C. 371©(2))

   a. ___ is transmitted herewith (required only if not transmitted by the International Bureau).

   b. X has been transmitted by the International Bureau.

   c. ___ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. X A translation of the International Application into English (35 U.S.C. 371 ©(2)).

7. X Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371©(3))

   a. ___ are transmitted herewith (required only if not transmitted by the International Bureau).

   b. ___ have been transmitted by the International Bureau.

   c. ___ have not been made; however, the time limit for making such amendments has NOT expired.

   d. X have not been made and will not be made.

8. ___ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371©(3)).

9. X An oath or declaration of the inventor(s) (35 U.S.C. 371©(4)).

10. __ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371©(5)).

Items 11. to 16. below concern other document(s) or information included:

11. X An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. X An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. X A FIRST preliminary amendment.
    ___ A SECOND or SUBSEQUENT preliminary amendment.

14. ___ A substitute specification.

15. ___ A change of power of attorney and/or address letter.

16. X Other items or information:

(i) Sequence Listing in paper form
(ii) Computer diskette containing the Sequence Listing in machine-readable form
(iii) Statement to Support Filing and Submission in accordance with 37 C.F.R. §§ 1.821-1.825
(iv) Request and Form PCT/IB/308
(v) English language translation of Receipt in The Case of an Original Deposit
(vi) Cover page of WIPO Publication No. WO 01/22833
(vii) PTO/SB/08A with copy of English language Search Report and 6 references

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.50 09/856640	INTERNATIONAL APPLICATION NO PCT/JP00/06528	ATTORNEY'S DOCKET NUMBER 38331-0002

17. ☒ The following fees are submitted: **CALCULATIONS** PTO USE ONLY

Basic National Fee (37 CFR 1.492(a)(1)-(5):

Neither international preliminary examination fee (CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....**$1000.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....**$860.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....**$710.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4).....**$690.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)..... ..**$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =				**$860.00**	
Surcharge of $130.00 for furnishing the oath or declaration later than 20 ___ 30 ___ months from the earliest claimed priority date (37 CFR 1.492(e))				$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	4 -20 =		X $18.00	$	
Independent Claims	1 -3 =		X $80.00	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				**$860.00**	
__ Applicants claim small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.				$	
SUBTOTAL =				**$860.00**	
Processing fee of $130.00 for furnishing English translation later the 20 ___ 30 ___ months from the earliest claimed priority date (37 CFR 1.492(f). +				$	
TOTAL NATIONAL FEE =				**$860.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				**$40.00**	
TOTAL FEES ENCLOSED =				**$900.00**	
				Amount to be: refunded	$
				charged:	$

a. **X** A check in the amount of $900.00 to cover the above fees is enclosed.

b. ___ Please charge my Deposit Account No. 08-1641 in the amount of $_____ to the above fees. A duplicate copy of this sheet is enclosed.

c. **X** The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-1641. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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NAME: MARVIN A. MOTSENBOCKER

REGISTRATION NUMBER: 36,614

DATE: MAY 23, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No: 38331-0002

Applicants: Akira TSUBOKURA *et al.*

Application No.: To be assigned

Filing Date: May 23, 2001

Title: A PIGMENT-CONTAINING SUBSTANCE FOR FEED ADDITIVES

PRELIMINARY AMENDMENT

Director of Patents
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, Applicants respectfully request that the following amendments be entered into the application:

IN THE SPECIFICATION

Page 12, at the end of the specification, delete the Sequence Listing filed with the application and insert the Sequence Listing submitted concurrently herewith.

IN THE CLAIMS

Please amend the claims as follows:

--3. (Amended) The pigment-containing substance for feed additives according to claim 1, characterized in that a DNA nucleotide sequence corresponding to 16S ribosomal RNA of the microorganism in the microorganism culture precipitate has at least 98% homology with the nucleotide sequence as shown in SEQ ID NO: 1.--.

Please add the following new claims:

--5. (New) The pigment-containing substance for feed additives according to claim 2, characterized in that a DNA nucleotide sequence corresponding to 16S ribosomal RNA of the microorganism in the microorganism culture precipitate has at least 98% homology with the nucleotide sequence as shown in SEQ ID NO: 1.

6. (New) The pigment-containing substance for feed additives according to claim 5, characterized in that the microorganism in the microorganism culture precipitate is E-396 strain or a mutant thereof.--.

REMARKS

Applicants submit this Amendment to delete the Sequence Listing filed with the application and to indicate the insertion point for the Sequence Listing filed concurrently herewith.

Upon entry of this amendment, claims 1 through 6 will be pending. Applicants request examination of the claims. A first office action on the merits is now awaited. Should there be any questions, the Examiner is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,

May 23, 2001
Date

Marvin A. Motsenbocker
Reg. No. 36,614

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Marked-up copy of Claim 3

3. The pigment-containing substance for feed additives according to claim 1 ~~or 2~~, characterized in that a DNA nucleotide sequence corresponding to 16S ribosomal RNA of the microorganism in the microorganism culture precipitate has at least 98% homology with the nucleotide sequence as shown in SEQ ID NO: 1.

SPECIFICATION

A pigment-containing substance for feed additives

Technical Field

The present invention relates to a pigment-containing substance for feed additives consisting of a microorganism culture precipitate which contains carotenoid compounds.

Background Art

"Carotenoid compounds" is a generic name for a group of pigment which have a long-chain polyene structure, the majority of which have a C40 tetraterpenoid, and present yellow, orange, red or purple color. More specifically it refers to the compounds such as β-carotene, astaxanthin, canthaxanthin, zeaxanthin, echinenone, adonirubin and adonixanthin. These compounds can be used as natural pigments useful for feed additives, food additives, pharmaceuticals etc. For example, astaxanthin is valuable from an industrial point of view as a feed additive such as a color improver for bred fishes such as salmon, trout, red sea bream etc., and also as a safe natural food additive. Adonixanthin is as promising as astaxanthin, as a feed additive, a food additive and pharmaceutical as the astaxanthin is, if its industrial production process can be established. Canthaxanthin has been used as a feed additive, food additive, cosmetic, etc., and zeaxanthin has been used as a feed additive and food additive. Further, echinenone and adonirubin also expected to be used as feed additives, food additives etc. As production processes for these carotenoid compounds, chemical synthesis, production by microorganisms, extraction from natural products etc. are known, and regarding astaxanthin and canthaxanthin, their chemical synthetic products are already on sale. Comparing with a chemical synthesis, the production process of carotenoid compounds by microorganisms is advantageous due to the high level of safety, since neither metal catalysts nor solvents are used therein. Generally, carotenoid compounds are unstable in the presence of oxygen and light. However, since carotenoid compounds produced by microorganisms are accumulated inside the cell of a microorganism, their stability is ensured by the cell membrane, cell wall and other antioxidants. When carotenoid compounds are used as feed additives or the like, a high level of storage stability is an advantage, and if the microorganism itself can be

used, this would be a great advantage. In contrast, if intercellular carotenoid compounds are used after being extracted and purified, it is necessary to used chlorine-containing solvents or the like which may be unsafe. Accordingly, the use of a microorganism culture precipitate consisting of a microorganism which contain carotenoid compounds as a feed additive has a great advantage. In the case where the microorganism producing carotenoid compounds has a hard cell wall, the absorption efficiency is extremely low if a natural cell is provided. So the cell should be disrupted mechanically or decomposed with chemical agents or enzymes, and this leads to a cost disadvantage. In addition, there is a problem that the stability of carotenoid compounds decreases after the cell is disrupted. Furthermore, when the content of carotenoid compounds existing in a microorganism culture precipitate is low, a large mount of precipitate is needed in the production process of the feed, and this causes other problems such as poor operability, high transport cost and nutritional inbalance of the feed. However, there is not known a pigment-containing substance consisting of a microorganism whose lack of a hard cell wall allows easy use of the accumulated carotenoid compounds; and, further consisting of a microorganism culture precipitate which contains at least 3 mass % carotenoid compounds.

Among carotenoid compounds, astaxanthin is a red pigment contained in Pisces such as salmon, trout, red sea bream etc., and Crustacea such as shrimps, crabs, etc., and it is useful because of its beautiful color, and as stated above, it has been broadly used as a feed additive and a natural food additive. Regarding the red yeast Phaffia rhodozyma, which is known to produce astaxanthin, there is a problem that, being a yeast, it has a hard cell wall. As processes for the production of astaxanthin by bacteria, the followings are known, but regarding each of them, the content per weight of dry cell is low: a bacterium Brevibacterium No.103 strain belonging to the genus Brevibacterium produces merely 0.003% of astaxanthin per weight of dry cell (*Journal of General and Applied Microbiology*, 15, 127, 1969). Also, another bacterium Paracoccus marcusii DSM11574 strain belonging to the genus Paracoccus produces only 0.022% of astaxanthin per weight of dry cell (WO 99/6586).

The object of the present invention is to provide a pigment-containing substance for feed additives consisting of a microorganism culture precipitate which contains a high concentration of carotenoid compounds useful as a natural pigment.

Disclosure of the Invention

In order to achieve the above object, the present invention provides the following means.

1. A pigment-containing substance for feed additives comprising a microorganism culture precipitate which contains at least 3 mass % carotenoid compounds.

2. The pigment-containing substance for feed additives, characterized in that at least 40 mass % of the carotenoid compounds is astaxanthin.

3. The pigment-containing substance for feed additives, characterized in that a DNA nucleotide sequence corresponding to 16S ribosomal RNA of the microorganism in the microorganism culture precipitate has at least 98% homology with the nucleotide sequence shown in SEQ ID NO: 1.

4. The pigment-containing substance for feed additives, characterized in that the microorganism in the microorganism culture precipitate is E-396 strain or a mutant, thereof.

This specification includes part or all of the contents disclosed in the specification of Japanese Patent Application No. 11-279337, which is a priority document of the present application.

The present invention is described more specifically as follows.

The pigment-containing substance for feed additives of the present invention comprises a microorganism culture precipitate. First, the microorganism culture precipitate is disclosed. The microorganism culture precipitate of the present invention can be obtained by a process wherein mainly the cell of microorganism producing carotenoid compounds is cultured to produce these compounds, the resulting culture is treated by filtration, centrifugation etc., followed by the removal of a certain amount of moisture. Any method for culturing a strain which produces carotenoid compounds can be used on the condition that carotenoid compounds are generated. For example, the following method can be adapted. As a medium, one which contains the followings is used: carbon sources, nitrogen sources, inorganic salt, which are necessary for the growth of bacteria producing carotenoid compounds, and particularly required

substances (e.g. vitamins, amino acids, nucleic acid bases etc.) as needed. The carbon sources to be used include carbohydrate such as glucose, sucrose, fructose, trehalose, mannose, mannitol and maltose; organic acids such as acetic acid, fumaric acid, citric acid, propionic acid, malic acid and malonic acid; alcohols such as ethanol, propanol, butanol, pentanol, hexanol and isobutanol; and the like. The ratio of added carbon sources depends on the type of carbon sources, but generally 1 to 100g per L of medium, preferably 2 to 50g is applied. The nitrogen sources to be used include one or more selected from potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulfate, diammonium hydrogen phosphate, ammonia, urea and the like. The ratio of added nitrogen sources depends on the type of nitrogen sources, but generally 0.1 to 10g per L of medium, preferably 1 to 3g is applied. The inorganic salt to be used includes one or more selected from potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium phosphate, magnesium sulfate, magnesium chloride, ferrous sulfate, ferrous chloride, manganous sulfate, manganous chloride, zinc sulfate, zinc chloride, cupric sulfate, calcium chloride, calcium carbonate, sodium carbonate and the like. The ratio of added inorganic salt depends on the type of inorganic salt, but generally 0.001 to 10g per L of medium are applied. The particularly required substances to be used include one or more selected from vitamins, nucleic acids, yeast extract, peptone, meat extract, malt extract, corn steep liquor, dry yeast, soybean cake, soybean oil, olive oil, corn oil, linseed oil and the like. The ratio of added particularly required substances depends on the type of the substances, but generally 1 to 200g per L of medium, preferably 10 to 100g is applied. The pH of the medium is 2-12, preferably it is adjusted to 6-10. As culture conditions, a shaking culture or an aeration agitation culture is carried out at 15-80°C, preferably at 20-35°C, ordinarily for 1-20 days, preferably for 2-8 days.

Next, moisture is removed from the culture obtained by the above method. The amount of moisture removed to obtain the pigment-containing substance for feed additives of the present invention depends on the state of the cultured medium (e.g. pigment content), but as a general process, first filtration is carried out, and when removal of moisture is further needed, drying of the precipitate follows. The methods of filtration include ordinary filtration, centrifugation and the like. Since the obtained precipitate contains water and precipitate comprising dessolved medium ingredients such as saline and carbohydrate, it is effective that water is added to the precipitate removed from the cultured medium, suspended, and then the precipitate is separate the precipitate again, so that the amount of carotenoid compounds therein

increases. By this process, the medium ingredients dissolved in water can be removed to some extent. In the case where the amount of carotenoid compounds should be increased, it is possible to apply a method for removing moisture by drying the precipitate. The methods for drying the precipitate include ordinary spray drying, drum drying, freeze drying and the like.

The microorganism culture precipitate obtained by the above method can be used successfully as a pigment-containing substance. For the purpose of preventing the decomposition of carotenoid compounds, antioxidants such as BHT (butylated hydroxytoluene), ethoxyquin and vitamin E may be added to the culture precipitate. Further, the surface of the microorganism may be covered with gelatin or the like.

Now, the microorganism used for the present invention is described. The microorganism used for the present invention is not specifically limited so long as it can produce carotenoid compounds by the culture of microorganisms such as bacteria and yeast, and can contain at least 3 mass % carotenoid compounds in its culture precipitate. However, considering the use of carotenoid compounds accumulated in the microorganism during the culture, it is preferable to employ a bacterium whose cell wall is thin enough to utilize the pigment effectively. In view of the growth speed and productivity of carotenoid compounds, it is especially preferable that a DNA nucleotide sequence corresponding to 16S ribosomal RNA is substantially homologous with the nucleotide sequence shown in SEQ ID NO: 1.

Taking the frequency of occurrence of errors in determining DNA nucleotide sequence into consideration, the term "substantially homologous" used herein means at least 98% homology.

In the bacteria having the sequence substantially homologous with the above sequence, carotenoid compounds such as astaxanthin, adonixanthin, β -carotene, echinenone, canthaxanthin, zeaxanthin, β -cryptoxanthin, 3-hydroxyechinenone, asteroidenone, adonirubin are accumulated by culture as a mixture. The ratio of generated carotenoid compounds contained in the cell can be changed, for example by changing aerobic culture conditions. By way of example, the ratio of generated adonixanthin can be changed by increasing the concentration of dissolved oxygen in culture medium. Also, the cell having an altered ratio of carotenoid compound production can be obtained by mutation. The methods of mutation include physical

methods such as X-ray irradiation and ultraviolet ray irradiation, the use of chemical mutagens, and artificial mutations wherein a chemical method of mutation such as treatment with NTG (N-methyl-N'-nitoro-N-nitrosoguanidine) and EMS (ethylmethane sulfonate).

Among the said bacteria, E-396 strain is one wherein astaxanthin makes up at least 40 mass % of the generated carotenoid compounds. This strain was newly isolated by the present inventors, and was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1 Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, Japan) under accession No. FERM BP-4283 on April 27, 1993. The mycological properties of this strain are disclosed in Japanese Patent Application Laying-Open (kokai) Nos. 7-79796, 8-9964 and 9-308481. A DNA nucleotide sequence corresponding to 16S ribosomal RNA of this strain is as shown in SEQ ID NO: 1.

Astaxanthin produced by the strain whose DNA nucleotide sequence corresponding to 16S ribosomal RNA is substantially homologous with the sequence specified by the present invention, is (3S, 3'S)-astaxanthin, and the purity is almost 100%. Astaxanthin presented in natural products such as crayfish, haematococuss, salmon, trout and red sea bream is known to contain a high rate of (3S, 3'S). In contrast, Phaffia rhodozyma is known to contain a high rate of (3R, 3'R) which has an absolute configuration which is the reverse of astaxanthin presented in the nature. The astaxanthin produced by the strain of the present invention is 100% (3S, 3'S)-astaxanthin and it is valuable from an industrial point of view that it has the same absolute configuration as the majority of astaxanthins in nature.

Some examples are provided below to describe the present invention more specifically, but the present invention is not limited thereto.

Best Modes for Carrying Out the Invention

Example 1:

A medium 6mL consisting of the composition of Table 1 was put into a test tube whose diameter is 18mm and a steam sterilization was carried out at 121°C for 15 minutes. One platinum loop of E-396 strain (FERM BP-4283) was inoculated thereinto, and a reciprocal shaking culture was carried out at 350 rpm at 28°C for 2 days. 2mL

of this culture was transferred to a 500 mL Sakaguchi flask containing 100mL of the medium which has the same composition as the above, and a reciprocal shaking culture was carried out at 100 rpm at 28°C for 6 days. Cells (wet weight 3.2g) were obtained from 100mL of the cultured medium by centrifugation. After the cells were added to 50 mL of ion-exchange water and fully suspended and centrifugation was carried out again to obtain cells (wet weight 3.1g). Then, 3.1g of the cells was freeze-dried to obtain 1.1g of dried cell. The carotenoid content existing in the dried cell was analyzed by high-performance liquid chromatography. The composition of carotenoid compounds is shown in Table 2. The water content in the dried cell was 2.5%.

Table 1

Composition	Added amount (g/L)
Yeast extract	20
Peptone	5
Sucrose	100
KH_2PO_4	1.5
$Na_2HPO_4 \cdot 12H_2O$	3.8
$MgSO_4 \cdot 7H_2O$	0.5
$FeSO_4 \cdot 7H_2O$	0.01
$CaCl_2 \cdot 2H_2O$	0.01
pH7 (adjusted to this value using Na_2CO_3)	

Table 2

Carotenoid compounds	Content (mg/g)
β -carotene	1.6
echinenone	1.9
3-hydroxyechinenone	0.9
canthaxanthin	2.3
adonirubin	5.6
astaxanthin	13.0
asteroidenone	0.6
adonixanthin	5.3
zeaxanthin	0.01
total carotenoid	31.2

Example 2:

E-396 strain (FERM BP-4283) was mutated with NTG (N-methyl-N'-nitoro-N-nitrosoguanidine) and intensely red colored colonies were selected. Carotenoid compounds in the cultured medium were analyzed, and mutant strains with increased astaxanthin yield were selected. The medium 5mL consisting of the composition of Table 1 was put into a test tube whose diameter is 18mm and a steam sterilization was carried out at 121°C for 15 minutes. One platinum loop of the mutant E-396 strain was inoculated thereinto, and a reciprocal shaking culture was carried out at 300 rpm at 30°C for 2 days. 2mL of this culture was transferred to a 500mL Sakaguchi flask containing 100mL of the medium which has the same composition as the above, and a reciprocal shaking culture was carried out at 120 rpm at 29°C for 2 days. Then, 800mL of this culture was inoculated into a 30L fermenter containing 20L of the medium consisting of the composition of Table 3, and an aerobic fermentation was carried out at 400rpm at 1.0vm at 29°C for 150 hours. Cells (wet weight 600g) were obtained from 18L of the cultured medium by Sharples centrifuge. After the cells were added to 20L of tap water and fully suspended, cells (wet weight 530g) were obtained by again using the same Sharples centrifuge. Then, after 1.5L of tap water was added and the cells (wet weight 530g) were fully suspended, and the cell was dried with a spray drier to obtain 200g of dried cell. As operating conditions, the air temperature was 210°C when introduced and was 105°C at exit, and the rate at which the suspension was fed was 38mL/min. The carotenoid content existing in the dried cell was analyzed by high-performance liquid chromatography. The composition of

carotenoid compounds is shown in Table 4. The water content in the dried cell was 3.1%.

Table 3

Composition	Added amount (g/L)
Yeast extract	20
Peptone	5
glucose	120
KH_2PO_4	1.5
$Na_2HPO_4 \cdot 12H_2O$	3.8
$MgSO_4 \cdot 7H_2O$	0.5
$FeSO_4 \cdot 7H_2O$	0.01
$CaCl_2 \cdot 2H_2O$	0.01
pH7 (adjusted to this value using Na_2CO_3)	

Table 4

Carotenoid compounds	Content (mg/g)
β -carotene	0.7
echinenone	1.1
3-hydroxyechinenone	0.6
canthaxanthin	1.7
adonirubin	5.4
astaxanthin	19.4
asteroidenone	0.8
adonixanthin	5.5
zeaxanthin	0.02
total carotenoid	35.2

Advantage of the Invention

Since the carotenoid existing in the pigment-containing substance for feed additives of the present invention is stabilized by the action of cell membrane, cell wall and the like of a microorganism, the pigment-containing substance for feed additives is resistant to oxygen, light and so on, and can be stably stored for a long time period.

All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

CLAIMS

1. A pigment-containing substance for feed additives comprising a microorganism culture precipitate which contains at least 3 mass % carotenoid compounds.

2. The pigment-containing substance for feed additives according to claim 1, characterized in that at least 40 mass % of the carotenoid compounds is astaxanthin.

3. The pigment-containing substance for feed additives according to claim 1 or 2, characterized in that a DNA nucleotide sequence corresponding to 16S ribosomal RNA of the microorganism in the microorganism culture precipitate has at least 98% homology with the nucleotide sequence as shown in SEQ ID NO: 1.

4. The pigment-containing substance for feed additives according to claim 3, characterized in that the microorganism in the microorganism culture precipitate is E-396 strain or a mutant thereof.

ABSTRACT

This invention provides a pigment-containing substance for feed additives consisting of a microorganism culture precipitate which contains a high concentration of carotenoid compounds. This pigment-containing substance for feed additives is resistant to oxygen, light and so on, and can be stably conserved for a long time period.

Attorney's Docket No.:_____

DECLARATION, POWER OF ATTORNEY AND PETITION

I (We), the undersigned inventor(s), hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I (We) believe that I am (we are) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

A PIGMENT-CONTAINING SUBSTANCE FOR FEED ADDITIVES

the specification of which

- ☐ is attached hereto.
- ☐ was filed on _____ as Application Serial No. _____ and amended on _____.
- ■ was filed as PCT international application Number PCT/JP00/06528 on September 22, 2000, and was amended under PCT Article 19 on _____ (if applicable).

I (We) hereby state that I (We) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; that I (We) do not know and do not believe that this invention was ever known or used before my invention or discovery thereof, or patented or described in any printed publication in any country before my invention or discovery thereof, or more than one year prior to this application, or in public use or on sale in the United States for more than one year prior to this application; that this invention or discovery has not been patented or made the subject of an inventor's certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months before this application.

I (We) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

I (We) hereby claim foreign priority benefits under Section 119(a)-(d) of Title 35 United States Code, of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Application No.	Country	Filing date	Priority claimed
279337/1999	Japan	September 30, 1999	■ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

I hereby claim the benefit under Section 119(e) of Title 35 United States Code, of any United States application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

I (We) hereby claim the benefit under Section 120 of Title 35 United States Code, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Section 112 of Title 35 United States Code, I (We) acknowledge the duty to disclose material information as defined in Section 1.56(a) of Title 37 Code of Federal Regulations, which occurred between the filing date of the prior application and national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented, abandoned)

6 And I (We) hereby appoint: Patricia D. Granados, Registration No. 33,683; John P. Isacson, Registration No. 33,715; Ronald J. Kamis, Registration No. 41,104; Susan E. Shaw McBee, Registration No. 39,294; Marvin Motsenbocker, Registration No. 36,614; and Colin G. Sandercock, Registration No. 31,298.

I(We) hereby request that all correspondence regarding this application be sent to the firm of HELLER EHRMAN WHITE & MCAULIFFE whose Post office address is: 1666 K Street, NW, Suite 300, Washington, DC 20006-1228 U.S.A.

I (We) declare further that all statements made herein of my (our) knowledge are true and that all statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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